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Correlation Between Mutations in p53 Gene and Protein Expression in Human Lymphomas

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A discordance between p53 protein overexpression and the presence of mutations in the gene has been observed in many types of tumors, including human lymphomas. To probe this finding, we have studied a large series of 94 lymphomas of different pathologic types and histologic differentiation. Analyzing exons 5–9, we have found mutations in the p53 gene in 7 of 94 cases distributed in different subtypes: 4/12 (33%) high-grade B-cell non-Hodgkin's lymphomas (B-NHLs), in 1 of 5 (20%) high-grade mucosa-associated lymphomas (MALT), in 1 of 22 (4.5%) anaplastic large cell lymphoma (ALCL), and in 1 of 24 (4%) T-cell NHLs. Immunostaining with anti-p53 antibody DO-7 was possible in 87 lymphomas, and overexpression of p53 protein was observed in 16 cases (18%). A discrepancy between the results of SSCP and immunostaining was detected on 18 tumor samples. Two cases with mutations in the gene showed no altered protein expression and 16 cases overexpressed p53 protein had no point mutations. In these cases, the possibility that mutations occur outside the exons studied has been tested and the entire coding sequence analyzed. Only one case showed a mutation in exon 10, and we found two cases carrying a polymorphism in exon 4 and in intron 10.

We conclude that mutations in p53 occur mainly in high-grade B-cell NHLs. Although not limited to a specific subtype of lymphoma, they may be rare in Hodgkin's disease and in low-grade lymphomas. The discrepancies between overexpression and presence of mutations suggest (1) the existence of another mechanism to stabilize the p53 protein, and (2) that the immunohistochemistry cannot be used to predict mutations in the gene. Am. J. Hematol. 55:1–8, 1997. © 1997 Wiley-Liss, Inc.

Key words: p53; mutations; protein overexpression; lymphomas

INTRODUCTION

The p53 gene is located on the short arm of chromosome 17 (17p13). It encodes a nuclear phosphoprotein that is involved in the control of the transition from G1 to S-phase in the cell cycle and has a negative effect on cellular proliferation. Recent studies show that p53 may play a role in the induction of apoptosis [1,2] and repair of DNA damage [3]. All these processes, in which p53 is involved, are thought to be mediated through the transcription factor activity of p53 protein [4].

p53 mutations can induce loss of the tumor suppressor function of the gene and then accelerate tumorigenesis. There is considerable evidence of deletion and mutations of p53 gene in a wide variety of human tumors [5]. The majority of mutations in p53 gene are missense mutations that occur in an evolutionary conserved region that includes exons 5–8 [6]. Wild-type p53 protein is present at low levels in the cell and has a short half-life. By contrast, these mutations induce conformational changes that result in an altered protein having a longer half-life (4–8 hr), leading to the accumulation of the mutated protein in the cell and making it possible to detect by immunohistochemical techniques.

Mutations on the p53 gene are not a very common event in non Hodgkin's lymphomas (NHLs) [7,8], however they appears in high frequency in NHL B cell lines [9]. By contrast, overexpression of p53 protein seems to be a frequent finding in these tumors, primarily in high-grade lymphomas [8,10–12].

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TABLE I. Series of Different Groups of Lymphoma Analyzed for Mutations in Gene and Protein Overexpression

Type of lymphoma	Cases studied for mutation in p53 gene (Mutated/total)	Analysis of expression of p53 protein (>10% positive cells/total)
MALT		
Low grade	0/11	2/11
High grade	1/5	4/5
ALCL	1/22	2/18
NHL B-cell		
Low grade		
Centrocytic	0/3	0/3
Centrocytic–centroblastic	0/4	0/4
Mantle lymphomas	0/1	0/1
Other types	0/2	0/1
High grade		
Centroblastic	2/5	2/5
Lymphoblastic	0/1	0/1
Burkitt	1/3	1/2
Other types	1/3	3/3
NHL T-cell		
Low grade	2/13	2/12
High grade	0/11	0/10
Hodgkin's disease	0/10	0/11
Total	8/94	Total 16/87

In our study, we looked for p53 gene mutations and protein accumulation in tumor cells in a large series of human lymphomas of different subtypes, in order to determine the involvement of these alterations in a specific group and to analyze the correlation between immunostaining and p53 gene mutations. Our results show that p53 mutations occur more frequently in high-grade lymphomas, and suggest the existence of other mechanisms of inactivation, to explain the discrepancies between immunohistochemistry and detection of mutations.

MATERIAL AND METHODS

In this study, we have analyzed a large, heterogeneous series of 94 lymphomas, including 10 Hodgkin's lymphomas, 16 MALT lymphomas, 22 ALCL, 24 T-cell NHLs, and 22 B-cell NHLs (Table I), obtained from the Pathology Department of the Jimenez Diaz Fundation and diagnosed according to Kiel and Isaacson classifications [13]. Genomic DNA was obtained from tumor samples and, when possible, from normal tissue, adjacent to the tumor, or peripheral blood.

LOH Analysis

We looked for allelic loss in the tumor, comparing it with normal tissue from 40 individuals. To verify whether the normal tissue selected from an area around the tumor was infiltrated by the tumor, previously we looked for monoclonal rearrangements amplifying the CDR II and CDR III regions of immunoglobulin heavy chain gene (IgH) or the γ chain of the T-cell receptor gene (TCR-γ) [14]. In all cases, the normal tissue showed a broad band (polyclonal population), indicating that they were not infiltrated by the tumor. The conditions for polymerase chain reaction (PCR) to examine CDR regions of the IgH and TCR-γ genes were previously described by Robledo et al. (1995) [15].

To determine LOH, using PCR, we amplified two polymorphic markers at 17p, a variable number of tandem repeats (VNTR) near the p53 gene (D17S30) [16], and an intragenic CA microsatellite [17]. A PCR-based assay to determine allelic losses in primary tumors may be subjected to the problem of contamination with normal cells. We have attempted to solve this problem, reducing the number of PCR cycles to make difficult the overamplification of the normal DNA from normal cells. Likewise, the percentage of malignant cells in the samples was estimated indirectly by immunohistochemical detection of the nuclear antigen Ki-67.

Polymerase Chain Reaction–Single-Strand Conformation Polymorphism Analysis

PCR-SSCP analysis to detect mutations was initially performed in all lymphomas in exons 5–9 of the p53 gene; in those cases showing p53 overexpression and carrying no mutations in these exons, the entire coding sequence of the gene was analyzed. The primers used have been previously described: exon 2–3 [18], exon 4 [19], exon 5 [7], exon 6 [20], exons 7 and 8 [21], exon 9 [22], exon 10 [19], and exon 11 [18].

Amplification was carried out in 10 μl of a reaction mixture containing 1× PCR buffer, 200 μM of dNTPs,

100 ng of DNA, 10 pmol of each primer, 1 unit of Taq polymerase and 1 μCi of α ^{32}P dCTP. The PCR cycle consists of 35 cycles of 30 sec at 94°C for denaturation; annealing temperatures were 56°C (exons 5 and 9), 74°C (exon 6), 64°C (exon 7), 62°C (exon 8), and an extension of 72°C for 1 min. The PCR products were diluted and mixed with a loading solution (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue).

After a denaturation the samples were loaded on 6–8% nondenaturing gels with 10% glycerol. Electrophoresis were performed at 10 W for 11–18 hours. The gel was then dried on filter paper and exposed to X-ray film at room temperature or 4°C for 1–6 hr.

To determine the sensitivity of our SSCP technique, we used DNA with a polymorphic variant and a normal DNA and mixed them at different ratios to obtain dilutions from 50–1% of the mutant DNA.

Direct Sequencing

All cases showing an abnormal pattern in SSCP were sequenced to characterize the mutations. Sequencing was done with the same primers used for SSCP analysis except for the exon 5, in which we used an internal sense primer 5′TACAGTACTCCCCTGCCCTCAA3′. The sequencing reactions were performed with the chain termination method [23], using the Sequenase Kit (USB, Cleveland, OH, USA, 70170). The products were then electrophoresed on a 6% denaturing polyacrylamide gel. All mutations were confirmed by sequencing the complementary strand.

Immunohistochemistry

Immunohistochemical staining of p53 was performed on paraffin-embedded sections, which were placed on poly-L-lysine-coated glass slides and dried at 56°C overnight. Deparaffinized and rehydrated sections were heated by pressure cooker in 0.01 M sodium citrate buffer pH 6 for 2 min, as a method for antigen unmasking.

Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxidase in distilled water for 5 min. The sections were incubated with anti-p53 monoclonal antibody (DO-7, 1:100; Dakopatts S.A., Copenhagen, Denmark) for 30 min. The antibody recognizes an epitope at the N-terminus of the human p53 protein and reacts with both wild-type and mutant proteins. The sections were then stained using streptavidin–biotin–peroxidase complex technique (Dakopatts). washing in TBS between steps. Peroxidase activity was detected using diaminobenzidine chromogen (DAB). The slides were counterstained with hematoxylin and mounted. Negative controls were treated identically, except that TBS was used instead of the primary. Positive control sections from a breast cancer with known diffuse positivity were also included.

p53 Immunostaining Assessment

Quantification of the expression of p53 protein was done with an Image Analyzer (CAS-200, Becton-Dickinson SA, Leiden, Belgium) with a proliferation index program that provides an accurate and objective method of assessment.

RESULTS

LOH Analysis

In 40 of 94 cases, it was possible to obtain normal tissue from the same patient from whom we conducted a study of allelic loss. We obtained 36 informative cases with at least one of the markers used. Only one case (L420) showed LOH with both markers used. A reduction in intensity of one allele in tumor samples has not been detected in any case, suggesting that LOH has not occurred in these samples. However, it is possible that in tumors with a percentage of proliferating cells lower than 50%, normal cells constitute more than 10% of total cells, and we cannot discount the possibility that LOH really occurred in these samples.

Detection of Mutations in the p53 Gene

All 94 lymphomas were screened for mutations in exons 5–9 and in the corresponding donor and acceptor splice sites, by the SSCP analysis. We found abnormal bands in 9 cases with different diagnoses (2 MALT, 2 centroblastic, 2 LNH-B, 1 ALCL, 1 Burkitt's lymphoma, and 1 T-lymphoma). Figure 1 shows some of the abnormal bands mobility shifts found in the SSCP analysis. We have found changes in all these exons studied, excluding exon 9. These tumors were then sequenced to characterize and localize the mutations (Table II). The mobility shifts in SSCP of two of these cases (L84 and L432) resulted in a silent base change at codon 213 that did not alter the function of the p53 protein, representing a common polymorphism previously described by other investigators [24].

Sequencing analysis of the remaining 7 cases showed single base pair missense mutations in all of them (Table II). Mutations found in codons 179, 248, and 273 correspond to some of the typical hotspots described in the p53 gene [5,24].

In cases showing altered bands by SSCP analysis, we also studied the normal tissue of each patient, when it was available. Only in cases L84 and L432 did the normal tissue also show altered bands in SSCP that correspond to the polymorphism mentioned above. In the other cases, mutant bands appeared only in the tumor, indicating that these changes are somatic mutations. SSCP analysis in all cases, except the case with LOH (L420), showed the normal pattern together with altered bands (Fig.1), indicating that mutations are present in

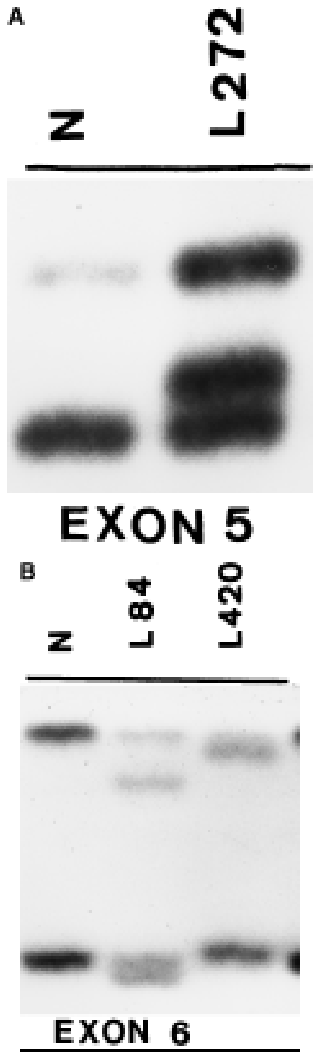

Fig. 1. SSCP analysis in exons 5 (a) and 6 (b). Cases with mobility shifts are shown in comparison with a normal pattern (N).

heterozygosity. In case L420, we only found altered bands corresponding to tumor cells (Figs. 1b, 2), confirming LOH and the existence of a mutation in the other allele. The mutations were found in NHL of different subtypes, but all of these were high-grade lymphomas.

Cases showing more than 10% positive cells and without mutations in exons 5–9 were then analyzed for mutations in the other exons (2, 3, 4, 10, and 11) of the gene. We have found three SSCP variants, one in exon 4 corresponding to a polymorphism that codes for either an arginine or proline at codon 72. It has been found in 10 of 16 of these cases. The other two were at exon 10: a missense mutation at codon 338 in a T-NHL, and an A-T change in intron 10 at 30 bp downstream of the 3′ boundary of exon 10 in a gastric MALT lymphoma. This polymorphism has previously been described [25].

Detection of p53 Overexpression

Immunohistochemical detection was performed using the anti-p53 mouse monoclonal antibody DO-7 in 87 of 94 lymphomas studied for the detection of p53 mutations. In these tumors, the estimated percentage of positive cells varies widely from 0% to 50%, and all positive cells showed nuclear staining (Fig. 3a–b). The expression levels of p53 protein are shown separately in Hodgkin's lymphomas and in different subtypes of NHLs (Table I); we have studied the expression pattern in 10 Hodgkin's lymphomas, 18 ALCL, 16 MALT, 20 NHL-B, and 22 LNH-T (Fig. 4a–e).

In NHLs we have found that the majority of low-grade lymphomas showed low levels of p53 protein (<10% positive cells), and most cases showing high levels of p53 protein correspond to high-grade lymphomas. In Hodgkin's lymphomas, all cases showed very low levels of the protein (<5%) (Fig. 4a).

In a comparative study of protein expression and p53 mutations, we that found the majority of cases (6 of 8) with mutations in the p53 gene showed a percentage of p53-positive cells over 10%, but two cases showed low-level had mutations; one case was a centroblastic lymphome with 4% positive cells, and the other was a T-cell NHL showing 0% of cells expressing p53 protein (Table II). Among the cases lacking p53 mutations, we have found 10 of 80 (12.5%) lymphomas in which p53 protein levels are high (>10%) and that do not correspond to alterations in the gene.

DISCUSSION

A p53 mutations is one of the most frequent alterations that occurs in human cancers of different types [5,26], including hematological malignancies [27,28]. The percentage of mutations in lymphoid malignancies varies in different histologic groups, with the highest values in adult T-cell leukemia (44%) [29] and Burkitt's lymphomas (37%) [7]. In previous studies done in NHLs, mainly of high grade, the frequency of p53 mutations, analyzing exons 5–8, are around 20% [30,31]. However, a recent study done by Kocialkowsky et al. [21] found mutations in NHLs outside the typical hotspots to be quite common.

We have studied a large, heterogeneous group of lymphomas, and found 8 of 94 cases with p53 mutations in exons 2–11 localized in 0 of 10 Hodgkin's disease patients, 1 of 22 ALCL, 1 of 16 MALT, 4 of 20 B-cell NHLs, and 2 of 24 T-cell NHL. Although a low frequency, if we consider only B-cell NHLs, the percentage of mutations is 20%, and increase to 33% (4/12) between B-cell NHLs of high grade, similar to that found by other investigators [30,31]. In Hodgkin's lymphomas, we have not found mutations, and the expression level of p53

TABLE II. Characterization of p53 Mutations

Case	Type	Exon	Codon	Change	p53 (%)	Ki67 (%)
L272	Burkitt	5	179	CAT-CGT	14.7	95
L79	T-cell NHL	6	214	CAT-CGT	0	<5
L420	B-NHL high grade	6	214	CAT-CGT	39.5	—
L361	MALT high grade	7	250	CCC-CTC	34.8	70
L382	Centroblastic	7	248	CGG-CAG	4.5	60
L405	Centroblastic	8	273	CGT-CAT	24.5	55
L350	ALCL	8	286	GAA-AAA	37.9	30
L155	T-cell NHL	10	338	TTC-CTC	47.7	<5

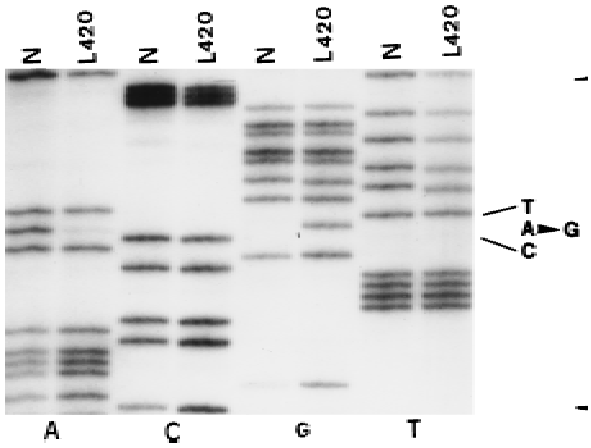

Fig. 2. Sequencing analysis of exon 6 amplified by PCR in case L420 in comparison with a normal sequence (N). This tumor had allele loss and the other allele presents a point mutation in codon 214 that results in a CAT-CGT substitution.

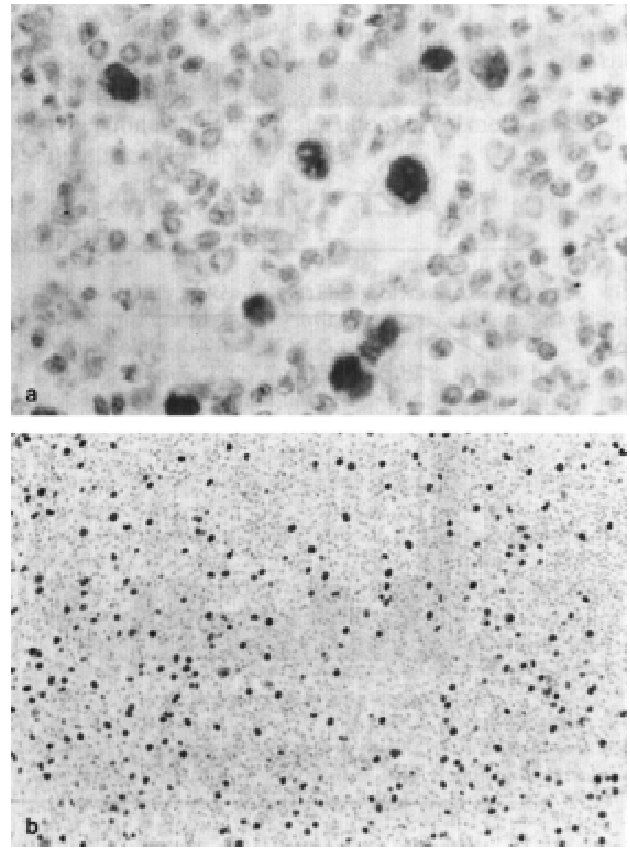

Fig. 3. a: Detail of the immunohistochemical detection of p53 protein by anti-p53 monoclonal antibody DO-7 in cells with positive nuclear staining. b: Case L350, an anaplastic large cell lymphoma showing 40% p53 positive cells. This case presents a mutation in codon 286 of exon 8.

protein was very low (<5%). It can be due to the large number of nontumor cells that appear with Reed-Sternberg cells in Hodgkin's disease. Other workers have reported a high proportion of Hodgkin's lymphomas showing overexpression of p53 protein, but they count only the Reed-Sternberg cells in the study [32]. So, our results can be representative of the participation of p53 in the different types of lymphomas: no mutations in low-grade NHLs, and alterations of this gene in high-grade NHLs, both nodal or extranodal, mainly of B-cell lineage.

The presence of mutations is thought to increase the half-life of the protein and to make detection possible by inmunohistochemistry. Thus, it could be an indirect method to detect mutations in the gene. However, there are a great number of discordant results between p53 overexpression and the presence of mutations in different tumors, including NHLs [11,33].

The range and distribution of percentages of p53 protein expression in all groups of lymphomas analyzed were similar (Fig. 4), with a majority of tumors exhibiting low expression (<10%) and the others showing more elevated percentages of positive cells (10–50%). In MALT and NHLs, this distribution could be correlated with the grade of malignancy of these tumors. Thus, high-grade lymphomas showed mainly high p53 protein expression: 4 of 5 high-grade out of 16 MALT lymphomas and 6 of 11 high-grade out of 20 B-cell NHLs presented protein overexpression. However, with regard to T-cell NHLs, only two different samples belonging to the same patient with a low-grade T-NHL presented overex-

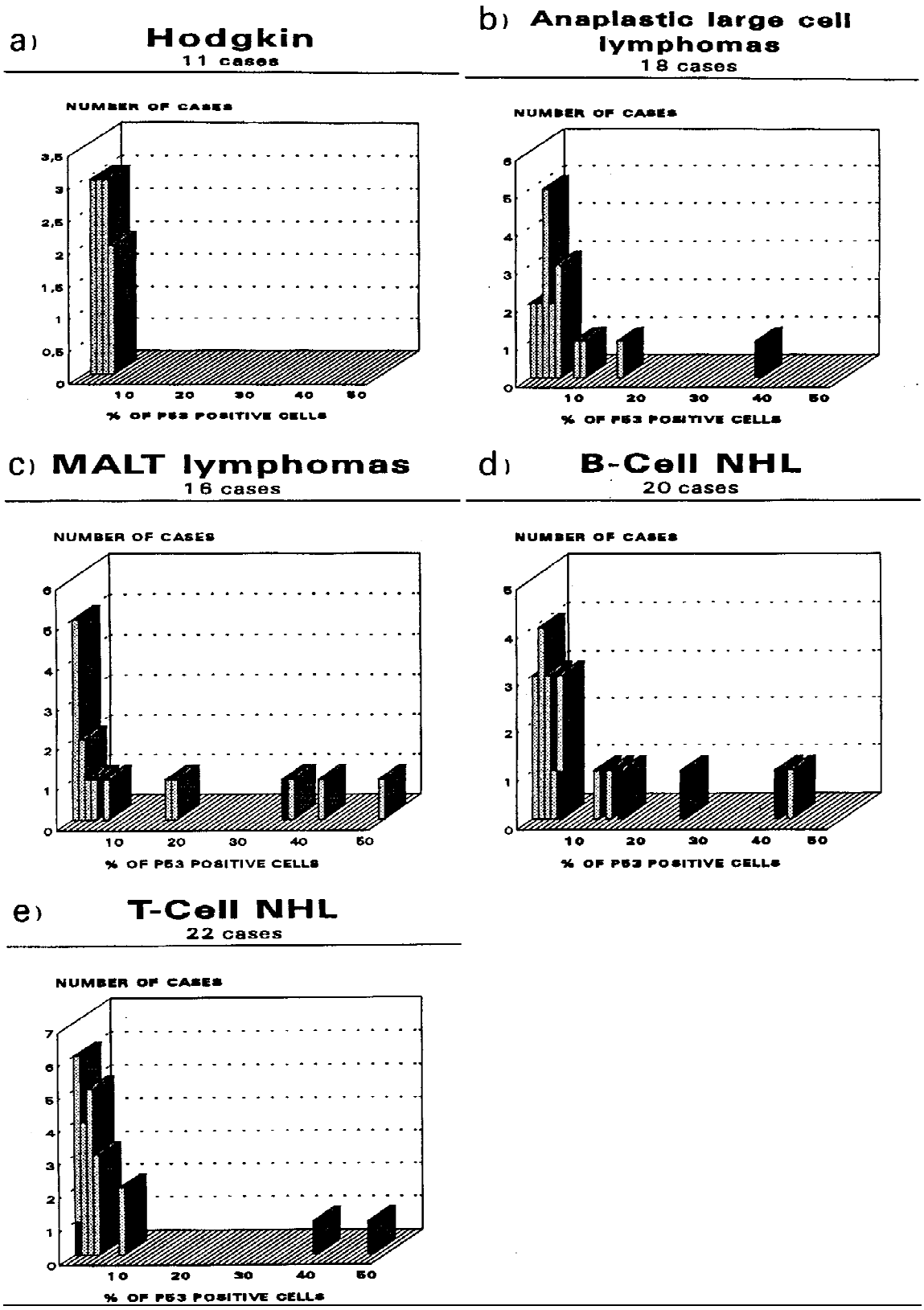

Fig. 4. Distribution of p53 protein expression levels in different histologic groups of lymphoma. Discrepancies between overexpression and presence of mutations in the gene can be seen. Cases with mutations are represented in black. a: All cases with Hodgkin's disease presented low expression level (<5% of positive cells). b: In anaplastic large cell lymphomas, only one case had a mutation in the gene and overexpressed the protein. c: In MALT lymphomas, there are six cases with more than 10% positive cells, only one of which presents a mutation. d: In B-cell NHLs, there is a case with mutation but low expression of p53 protein. The other mutated cases overexpressed p53. e: Two T-cell NHL have mutations, one presenting 0% p53 expression; the other overexpressed the protein.

pression of the protein and carried a mutation in exon 10 (Table II).

Combined analysis of p53 protein expression and mutations in the gene showed that there was not a total correlation. A discordancy in results of these two methods was observed on 18 of 87 (19%) cases. In two cases, the presence of mutations was not correlated with overexpression of the protein; the other 16 showed high levels of p53 protein, but mutations were not detected in the analysis of exons 5–9. In these cases, we have studied the entire coding sequence; only one case showed a mutation outside this conserved region: exon 10 (Table II). Mutations on exons 4 and 10 have also been demonstrated in lymphomas, by other investigators [21]. The absence of mutations in the remaining 15 cases suggests that more than one mechanism stabilizes the protein.

In another case with a high level of p53 protein, we have found a base change in intron 10; however, it is not probable that it has an effect on overexpression of the protein. We have also detected a polymorphism in codon 72 of exon 4 that changes an arginine for a proline, in a high proportion of these cases (10 of 16); however, no significant differences have been found in the distribution of this proline isoform in cases with high or low levels of p53 protein. Other investigators have attempted to find an association between this polymorphism and disease status, but they have not been successful [34,35].

Another possible explanation of the discrepancies between protein detection and presence of mutations is that SSCP is not 100% sensitive. In our hands, the PCR-SSCP technique can detect 5–1% of the mutant DNA when the abnormal and normal bands run well separated in the gel, and at least 10% of abnormal DNA when mutant bands migrate close to normal bands. This sensitivity is similar to that reported by other workers [33]. Also we have tried to increase this sensitivity by running the samples under two different conditions.

An alternative possibility is p53 inactivation by binding to other cellular proteins, such as mdm2, stabilizing the wild-type p53 protein. This inactivation independent of mutation has been demonstrated in other types of tumors [36].

In addition, not all cases carrying missense mutations showed high levels of positive p53 cells after immunostaining [31,33]. We found two cases (L79 and L382, Table II) carrying missense mutations and presenting 0% or low p53 protein expression. It has been proposed that nonsense or frameshift mutations may lead to the production of a truncated protein that is not possible to detect by immunohistochemistry. However, we found only missense mutations in these tumors. It is interesting to note that we found two cases with the same mutation, one showing overexpression and the other showing no p53 expression, that could be explained by another event, different from mutations, that could be responsible for the overexpression. Another explanation is that mutations in these lymphomas do not lead to the protein stabilization. It is also possible that DO-7 antibody is unable to recognize all types of mutated proteins.

In conclusion, we found that mutations on p53 gene in lymphomas are rare in low-grade NHLs, but they appear more frequently in B-cell NHLs. The fact that mutations occur in high-grade lymphomas suggests an association with tumor progression, as has been proposed by other investigators, [37–39]. Also, overexpression of p53 protein occurs mainly in high-grade lymphomas. The discordance between overexpression of the protein and mutations found in the gene suggests that another mechanism distinct to mutations is responsible for the accumulation of p53 protein in lymphomas. More work must be done to investigate alternative mechanisms to stabilize the p53 protein in cells.

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